REMARKS

A. Disposition of Claims

Claims 20 and 22-26 are pending in this application. Claim 23 is withdrawn from consideration as not readable on the elected species. Claims 1-19, 21, and 27-30 have been canceled without prejudice as being drawn to non-elected subject matter. The title has been amended to be more descriptive. The provisional rejection under 35 USC 101 as claiming the same invention as that of claims in copending Application No. 10/866,193 is appreciated as being a *provisional* double patenting rejection because any conflicting claims have not in fact been patented. There is no prior art. Reexamination and reconsideration of the application, as amended, are respectfully requested.

B. Compliance with 35 USC 112/1

The Patent Office rejected the claims under 35 USC 112/1 as failing to meet the enablement requirement. Under MPEP 2164, the test for enablement is whether one skilled in the art could make or use the subject matter defined by the claims without undue experimentation. Under MPEP 2164.01(a), the Wands factors are to be considered in determining whether any necessary experimentation is undue. Here, the specification is enabling with respect to the claimed subject matter.

i) First, there is considerable direction and guidance in the specification with respect to how to make and use the subject matter defined in the claims.

- **The specification describes the measures required to make and use the claimed embodiment at 12:1 – 14:21:**

> An altogether different method to achieve the desired result is to genetically engineer a phage so that it expresses molecules on its surface coat, where said molecules antagonize, inactivate, or in some other manner impede those actions of the HDS that would otherwise reduce the viability of the administered phages. One of the ways to accomplish this is to engineer a phage to express molecules that antagonize one or more of the complement components.

Complement components fix to bacteriophages, and these bacteriophages then adhere to certain white blood cells (such as macrophages) that express complement receptors. Numerous peptides have been synthesized that antagonize the functions of the various complement components. [See e.g. Lambris, J. D. et al, "Use of synthetic peptides in exploring and modifying complement reactivities" in Activators and Inhibitors of Complement, ed. R. Sim, Kluwer Academic Publishers, Boston, 1993.] Lambris et al. (op.cit.) cite "a series of synthetic peptides spanning the covertase cleavage site in C3 (that are) found to inhibit complement activation by both the classical and alternative pathways". Among the peptides cited is a six amino acid peptide (LARSNL, residues 746-751 of C3) that "inhibits both pathways equally well".

In one method of genetically engineering such a phage, a fusion protein is obtained, wherein the peptide will be bound to the carboxyl end of the surface protein of interest [See e.g. Sambrook, J., Fritsch, E., and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989]. This construct is made by cloning the gene for the phage surface protein into a plasmid vector system, and then cloning the oligonucleotide for the peptide of interest into this carrying vector by in-frame fusion at the 3'-end of the gene for the surface protein. This fusion of the gene for the phage surface protein with the oligonucleotide for the complement-antagonizing peptide would then be incorporated into the phage of interest by the in vivo generalized recombination system in the host bacteria for the phage of interest. Phage whose genomic sequence is already completely known, and phage whose genomic sequence is unknown or partially unknown can be used in the present invention.

The surface expression of a recombinant complement-antagonizing peptide is but one example of several complement-related strategies that might be used for these purposes. Another example would be the expression of a human complement-antagonizing protein on the surface of a phage. Several transplantation research facilities are currently expressing such human

complement-antagonizing proteins in transgenic animals, in the hopes that when these transgenic organs are donated they will not be immunologically rejected by a human recipient. [See e.g. Genetic Engineering News, Oct. 15, 1993, p.1.] In an analogous manner, the expression of such recombinant human complement-antagonizing proteins on the surface of a bacteriophage may allow the phage to delay being inactivated by the host defense system.

In addition to complement-related strategies, there are many other categories of molecules that can be recombinantly engineered into a phage to delay inactivation by the host's defense system. Other categories of molecules that could be expressed on the surface of bacteriophages, and would fall under the scope of this invention, include but are not limited to: interleukins and other cytokines; autocrines; and inhibitors of the various cellular activating or inhibiting factors (e.g. inhibitors of macrophage activating factor). Genes for these proteins (or for active subunits of them) can be incorporated into a phage genome so that they will be expressed on the surface.

In addition, if it were possible to get a given bacterial host strain to glycosylate a recombinant protein, then the purpose of the invention could be served by introducing genes that will express glycosylated proteins. Such proteins are known by their negative charge to repel immune cells, such as the macrophage. Examples might include but would not be limited to (1) the C-terminal portion of the β-subunit of human chorionic gonadotrophin (hCG), and (2) the various glycophorins on the surfaces of blood cells.

Phage modified in this manner are referred to as "anti-HDS engineered".

- **The specification provides the prophetic examples of Examples 4-6:**

Example 4

Genetic engineering of phage to express molecules that antagonize the host defense system, thereby enabling the phage to delay inactivation by the host defense system

Part 1. Making the Fusion Protein

Step 1. A double-stranded DNA encoding the complement antagonizing peptide LARSNL is synthesized on an automated oligonucleotide synthesizer using standard techniques.

Step 2. The gene for the phage coat surface protein of interest (see part 2, below) is cloned into a plasmid vector system, by techniques known in the art. The oligonucleotide that has been prepared in Step 1 is cloned into the plasmid vector system by in-frame fusion at the 3'-end of the gene for the surface protein.

Step 3. The fusion gene is then incorporated into a phage by the in vivo generalized recombination system in the host bacteria for the phage. The phage then expresses the fusion protein on its surface.

Part 2: Selecting phage coat surface proteins for fusion with the peptide/protein of interest.

A. Incorporating the gene for the complement-antagonizing peptide into a phage whose genome is well characterized

The orfx gene, which encodes a carboxy-terminal tail protein of lambda coliphage, is one for which it is known that foreign nucleotide sequences can be introduced without there being disruption of the structure or function of the phage. The tail surface protein expressed by the orfx gene is made into a fusion protein with the complement-antagonizing peptide, by the plasmid vector method described in part 1 above.

B. Incorporating a gene for a complement-antagonizing peptide into a phage whose genome is not well characterized.

Step 1. Selection of the phage surface protein to be fused with the complement-antagonizing peptide:

a) Isolation of phage coat surface proteins and preparation of antibodies thereto:

(1) Samples of the phage of interest are broken up in 0.1% SDS detergent for 2 minutes at 95°C. The mixture is cooled and placed in 9M urea, and is then separated by high resolution 2D gel electrophoresis. The protein fragments are then isolated from the gel, and processed as described below.

(2) Samples of the protein fragments from the gel are injected into animals to produce either polyclonal or monoclonal antibodies.

(3) Antibodies are isolated and then marked with uranium. These marked antibodies are reacted against whole phage. The marker pinpoints precisely those proteins on the surface of the phage to which the antibodies have bound through visualization by electron microscopy. [See e.g. K. Williams and M. Chase, ed., Methods In Immunology and Immunochemistry, Vol.1, 1967, Academic Press.] Antibodies directed against a surface protein extending outward from the surface of the virus are retained for further use.

b) Preparation of phage restriction fragments:

The genome of the phage is cut by restriction enzymes, and the resulting restriction fragments are cloned into expression vector plasmids. Each of these plasmids expresses its corresponding protein, creating a pool of expressed proteins.

c) Reacting the expressed proteins with the marked antibodies:

The antibodies directed against a surface protein extending outward from the surface of the virus are reacted against the proteins expressed by the plasmid vectors.

d) Correlating coat protein antibodies to the plasmid vectors that express the genes for those coat proteins:

The reaction of a marked antibody with an expressed protein pinpoints the expression plasmid whose enclosed restriction fragment expresses the particular protein. Thus, the genomic fragment encoding each coat surface protein is determined using the marked antibodies.

e) Determining that the gene in its entirety has been obtained:

The restriction fragments containing a gene for a surface protein are micro-sequenced by the Sanger technique to determine

(1) the precise amino acid sequence of the coat surface proteins;

(2) the presence of a start and a stop signal (indicating that the gene in its entirety has been obtained); and (3) the presence of either a C-terminal or an N-terminal amino acid.

Step 2. Fusing the candidate phage surface protein with the complement-antagonizing peptide of interest:

a) Preparing the coat protein gene for fusion:

The gene for a surface protein is contained in its plasmid expression vector. The oligo-nucleotide for the complement-antagonizing peptide is spliced into this plasmid expression vector by in-frame fusion at the 3'-end of the coat surface protein.

b) Incorporating the fusion gene into the phage of interest:

The fusion gene is incorporated into the phage by the in vivo generalized recombination system in the host bacteria for the phage.

c) Demonstrating that the phage expresses the fusion protein:

The phage is incubated with a corresponding heavy metal-marked antibody that has been raised against the coat surface protein. The marker is detected on the phage by electron microscopy only if the phage has expressed that fusion protein on its surface. [See e.g. K. Williams and M. Chase, Methods In Immunology and Immunochemistry, Vol.1, 1967, Academic Press.]

Example 5

Demonstration that the genetically engineered phage delay inactivation by the HDS, compared to wild-type phage

Two groups of mice are injected with phage as specified below:

Group 1: The experimental group receives an IV injection consisting of $1X10^{12}$ of the genetically modified phage, suspended in 0.5 cc of sterile normal saline.

Group 2: The control group receives an IV injection consisting of $1X10^{12}$ of the wild-type phage from which the genetically modified phage were derived, suspended in 5 cc of sterile normal saline.

Both groups of mice are bled at regular intervals, and the blood samples assayed for phage content (by pfu assays) to determine the following:

1) Assays for half-lives of the two phages: For each group of mice, the point in time is noted at which there remains in circulation only half (i.e., $1X10^{6}$) the amount of phage as administered at the outset. The point in time at which half of the genetically modified phage have been eliminated from the circulation is at least 15% longer than the corresponding point in time at which half of the wild-type phage have been eliminated from the circulation.

2) Assays for absolute numbers: For each group of mice, a sample of blood is taken at precisely 1 hour after administration of the phage. The criterion used is that at 1 hour post-injection, pfu assays reveal that the numbers of genetically engineered phage still in circulation in the experimental animal are at least 10% higher than the numbers of wild-type phage still in circulation in the control animal.

Example 6

Determination that the genetically engineered phage has a greater capacity than wild type phage to prevent lethal infections in mice.

Part 1. Peritonitis Model:

An LD_{50} dosage of E. coli is administered intraperitonally (IP) to laboratory mice. The strain of E. coli used is one known to be lysed by the coliphage strain that has been genetically engineered. The treatment modality is administered precisely 20 minutes after the bacteria are injected, but before the onset of symptoms. The treatment modalities consist of the following:

Group 1: The experimental group receives an IP injection consisting of $1X10^{12}$ of the genetically engineered lambda coliphage suspended in 2 cc of sterile normal saline.

Group 2: A first control group receives an IP injection consisting of $1X10^{12}$ of the wild-type phage from which the genetically modified phage were developed, suspended in 2 cc of sterile normal saline.

Group 3: A second control group receives an IP injection of sterile normal saline.

Evidence that treatment with the genetically modified phage prevented the development of a lethal event in the peritonitis model is measured by using the following three criteria:

(1) Survival of the animal

(2) Bacterial counts: Samples of peritoneal fluid are withdrawn every 1/2 hour from the three groups of infected mice, and the rate of increase or decrease in E. coli colony counts in the three groups is noted

(3) Phage control: Using the samples of IP fluid withdrawn from the infected mice, the numbers of pfu of the genetically engineered phage versus the numbers of pfu of the wild-type phage are noted.

Part 2. Bacteremia Model:

An LD_{50} dosage of E. coli is administered intravenously (IV) to laboratory mice, where the strain of E. coli used is known to be lysed by the coliphage strain that was genetically engineered. The treatment modality (see below) is administered precisely 20 minutes after the bacteria are injected, but before the onset of symptoms. The treatment modalities consist of the following:

Group 1: The experimental group receives an IV injection consisting of $1X10^{12}$ of the genetically engineered lambda coliphage suspended in 0.5 cc of sterile normal saline.

Group 2: A first control group receives an IV injection consisting of $1X10^{12}$ of the wild-type phage from which the genetically engineered phage were developed, suspended in 0.5 cc of sterile normal saline.

Group 3: A second control group receives an IV injection of 0.5 cc of sterile normal saline.

Evidence that treatment with the genetically engineered phage prevented the development of a lethal event in the bacteremia model is measured using the following three criteria:

(1) Survival of the animal

(2) Bacterial counts: In the samples of blood that are withdrawn every 1/2 hour from the three groups of infected mice, the absolute numbers as well as the rate of increase or decrease in E. coli colony counts is noted, for each of those three groups.

(3) Phage counts: In the samples of blood withdrawn from the infected mice, the numbers of pfu of the genetically engineered phage and the numbers of pfu of the wild-type phage are noted.

ii) Second, there was a high level of skill in the art at the time the application was filed. The level of skill in the molecular biology art was that of a postdoctoral fellow working in the laboratory. *Amgen Inc. v. Hoechst Marion Roussel Inc.*, 57 USPQ2d 1449, 1518 (D. Mass. 2001). Thus, the level of skill in the art was high.

iii) Third, all of the methods needed to practice the invention were well known. As of the 5 April 1994 priority date, for guidance regarding such conditions, see, for example, Sambrook, et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and Wiley & Sons, Inc., New York.

iv) Per MPEP 2164.01(a), the In re Wands Court held that the specification was enabling with respect to the claims at issue and found that "there was considerable direction and guidance" in the specification; there was "a high level of skill in the art at the

time the application was filed;" and "all of the methods needed to practice the invention were well known." Similarly, here, as indicated above, there was considerable direction and guidance in the specification; there was a high level of skill in the art at the time the application was filed; and all of the methods needed to practice the invention were well known. Additionally, the post-filing date inventor-created art of **Merril et al., Proc Natl Acad Sci 93: 3188 (1996), of record**, demonstrates that long-circulating bacteriophage that function as antibacterial agents can be isolated by a serial-passage technique and the relevant mutation in a capsid protein identified. Moreover, the post-filing date inventor-created art of **Vitiello et al., Virus Res 114: 101 (2005), attached**, establishes that the amino acid substitution in a capsid protein that enhances phage survival can be incorporated into a wild type background. Thus, here, considering all the factors related to the enablement issue, it must be concluded that it would *not* require undue experimentation to make and use the subject matter defined in the claims. The conclusion is the claims are in compliance with 35 USC 112/1 as meeting the enablement requirement.

CONCLUSION

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration and withdrawal of all outstanding rejections are respectfully requested. Allowance of the claims at an early date is solicited. If any points remain that can be resolved by telephone, the Examiner is invited to contact the undersigned at the below-given telephone number.

Respectfully submitted,

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An amino acid substitution in a capsid protein enhances phage survival in mouse circulatory system more than a 1000-fold

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Abstract

In experiments with germ free mice, free from adaptive antibodies to the bacterial virus λ phage, titers of the virus in the circulatory system have been reported to decrease by more than 10^9 pfu within 48 h of intraperitoneal intravenous or oral administration. Based on these observations, serial passage techniques have been used to select λ phage mutants, with 13,000–16,000-fold greater capacity to remain in the mouse circulatory system 24 h after intraperitoneal injection. In these prior studies the "long-circulating" phage, designated λArgo phage, had at least three mutations including one in the major phage capsid (E) protein, which resulted in the change of glutamic acid to a lysine at residue 158. In the current study, we demonstrate that this single specific substitution in the E protein is sufficient to confer the "long-circulating" phenotype. The isogenic pair of phage developed in this study consisting of the long-circulating marker-rescued λArgo phage, and the parental wild type phage can be used for studies of viral recognition mechanisms of the innate immune system and for the development of more effective antibacterial therapeutic phage strains.
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Early experiments involving the parenteral administration of phage led to observations of a rapid decrease in phage titers in the mammalian circulatory system. In one such experiment, published in 1934, a *staphylococcus* phage was found to decrease four orders of magnitude 5 min after intravenous (i.v.) injection and by 2 h a 7-log decline in titer was recorded (Nungester and Watrous, 1934). In other experiments, conducted in the 1930s, phage administered by intravenous inoculations were observed to accumulate an organ of the reticulo-endothelial system (RES), the spleen (Evans, 1933). The relative roles of the organs of the RES in this process were determined three decades later by Inchley (1969) who by using ^{51}Cr-labelled phage observed that the liver phagocytosed more than 99% of the phage introduced into the circulatory system (Inchley, 1969). However, the fate of phage introduced into the circulatory system in mammals was shown to be more complex by the observations of Evans (1933) and Geier et al. (1973) that phage taken up by the spleen remained fully functional and capable of replicating in bacteria days after their initial inoculation into the mammalian circulatory system. The mechanisms underlying the initial rapid and robust mammalian capacity for reducing phage titers in the circulatory system appear to be independent of the adaptive immune systems as the effect was observed in animal experiments in which the phage used were neoantigens. In these experiments, utilizing germ free mice with no detectable pre-existing adaptive antibodies to lambda phage, phage titers in the circulatory system decreased exponentially by more than 10^9-fold within 48 h of intraperitoneal (i.p.), intravenous or oral phage administration (Geier et al., 1973).

The recognition that components of the innate immune system, including the RES, can remove a significant proportion of administered phage led to the development of a serial passage selection method to isolate phage mutants with a greater capacity to remain in the circulatory system of the mouse (Merril et al., 1996). This method was used to isolate lambda phage mutants with a 13,000–16,000-fold greater capacity. Two "long-circulating" λ strains, λArgo1 and λArgo2, were isolated, both of which had at least one

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newly acquired mutation conferring a change of a glutamic acid to a lysine at residue 158 of the lambda capsid E protein (Merril et al., 1996). This mutation was designated as *argo1* mutation. The parent of the λArgo phages also contained two additional mutations, *cI90* and *c17*, each one of which confers clear plaque morphology on bacterial hosts. The current experiments were designed to determine whether the *argo1* mutation alone is responsible for the "long-circulating" phenotype.

1. Development of isogenic parental and mutant long-circulating Argo phage

To construct an isogenic pair of phage, consisting of a long-circulating wild type and a phage derived from the wild type with a single mutation in the E gene, we rescued the *E* gene *argo1* mutation, from the λArgo1 phage and then incorporated this mutation into a wild type background, as described below. This recombinant phage and its wild type parent were then used in animal clearance studies in which they were compared with the original λArgo1 phage, which was developed by selection.

The strain of *E. coli* used in this study was *E. coli* K-12 btuB::Tn10 from the NIH stock collection and the phage strains were W60 lcI60cY17 from the NIH stock and Argo1 lcI60cY17*arg1* and Argo2 lcI60cY17*arg2* as described previously (Merril et al., 1996). Bacteria and phage were grown in LB media. Phage stocks for injection into animals were grown in *E. coli* K-12 btuB::Tn10 to high titers using standard procedures (Silhavy et al., 1984). Large-scale preparations and purifications of bacteriophage by cesium chloride density centrifugation were performed according to Sambrook et al. (1989). Six-week old female BALB/C mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and the animal experiments were performed at Biocon Laboratory (Rockville, Maryland) with an approved animal protocol A0509b-c.

The λArgo1 phage carries at least three mutations, *cI90*, *c17* and *argo1*. To construct a λ phage with only the *E* gene mutation of λArgo1 phage in a wild type background without *cI90* and *c17* mutations, we first amplified the full length *E* gene from Argo1 strain by PCR, using primers, which incorporated *Eco*R1 and *Bam*H1 restriction sites. This amplification product was then ligated into pUC19 (New England Biolabs) using procedures described in Sambrook et al. (1989). The plasmid carrying the insert, pMAP, was then transformed into DH5-alpha competent cells (Invitrogen); the purified transformed cells were grown to an A_{590} of 0.3. The culture was then infected with a $\lambda E_{amb4}cI_{857}$ mutant, a capsid defective mutant that can only grow on a permissive *E. coli supE* host (C600). The cI_{857} mutation confers a turbid plaque phenotype at 32° and a clear plaque phenotype at 42°. $\lambda E^{+}cI_{857}$, recombinants were detected by plating the crossed phage lysate on mixed layer agar plates, containing the permissive *E. coli* (C600) and a non-permissive *E. coli* (MG1655) host at 42° C. The E^{+} phage that grew on both *E. coli* hosts were clear as opposed to E_{amb} phage, which grew only on the permissive *E. coli* host and were turbid. Clear plaques were purified and grown to high titers. DNA was extracted from these phages with the Promega Lambda DNA Extraction Kit. The presence of the *E* mutation in E^{+} phages was confirmed by DNA sequencing. Out of five plaques purified, two contained the *argo* mutation in the *E* gene. One of the two, designated marker-rescued lambda *argo1*, was chosen to test the long-circulating phenotype. Large scale preparations (4 l) of the parental lambda cI_{857}, the original *argo1* isolate from the λ*cI9*, c*Y17* strain, and the marker-rescued λ*argo1* phage were prepared by PEG precipitation and cesium chloride density centrifugation.

To compare the circulation times of the phage in mice three groups consisting of five female BALB/C, 6-week old, mice were each injected with one of the three-phage strains described above. A hundred microlitres of 2×10^{12} pfu/ml of phage were injected i.p. into each mouse, which provided for initial titers of 2×10^{11} pfu/ml in the mouse circulatory system. Blood samples (100 μl) were collected via orbital bleeds in heparinized tubes at 1 h, 5 and 24 h post injection. Phage titers were determined by serially diluting blood samples in tris-Mg-gelatin buffer, pH 7.0 (10-fold dilutions) and plating these dilutions on LB agar plates with 200 μl of Plat-

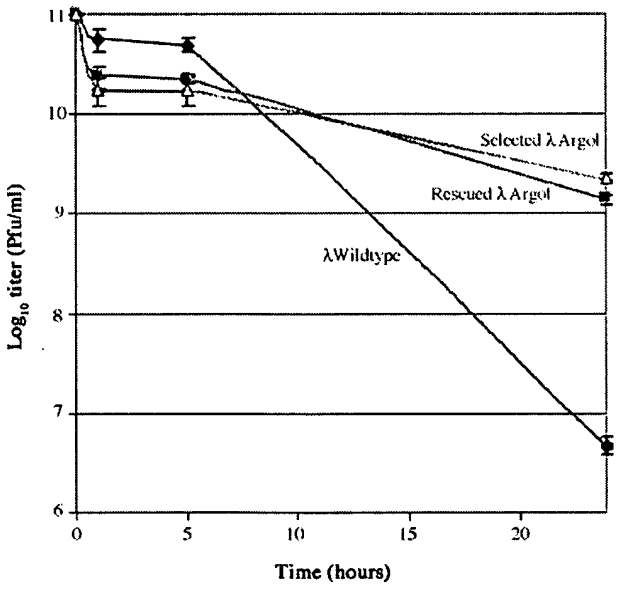

Fig. 1. Circulatory fate of λ phage. Three groups consisting of 5 female BALB/C mice (6-week old) were each injected with one of the three-phage strains described above (100 μl of 2×10^{12} pfu/ml phage were injected i.p. into each mouse) which provided for initial titers of 1×10^{11} pfu/ml in the mouse circulatory system. Phage titers in the circulatory system were determined sampling phage titers using orbital bleeds. The standard errors of the mean are indicated by the error bars for each of the data points. The marker-rescued λArgo1 showed a phenotype identical to the original λArgo1 phage; at the 24 h mark, both of these "long-circulating" phages remained in the blood stream at a concentration 4 logs greater than that of the corresponding wild type strain.

ing bacteria (HT115) (A_{590} = 0.3) and 3 ml .07% top agar. As shown in Fig. 1, the rescued λ*Argo1* showed a phenotype identical to the original λ*Argo1* phage; at the 24 h mark, both phages remained in the blood stream at a concentration of more than 4 logs higher than that of the corresponding wild type phage strain. The presence of a single mutation in the rescued lambda Argo phage was confirmed by DNA sequencing of the entire phage genome. Our results demonstrate that a single amino acid substitution, the substitution of the basic amino acid lysine for the acidic amino acid glutamic acid at position 158 of the lambda capsid E protein, which is present in about 450 copies per virion (Daniels et al., 1983), is sufficient to reduce the capacity of the mammalian innate immune system to diminish that phage strain's concentration in the circulatory system and that no other mutations are needed for this phenotype.

2. Concluding comments

In recent experiments, utilizing T7 phage, the rapid elimination of T7 phage from the circulatory system of the mouse was reported to be greatly diminished in immunocompromized B-cell deficient mice (Srivastava et al., 2004). As B-lymphocytes secret "natural" antibodies that play a significant role in the innate immune response, it may be useful to study the phenotype of the isogenic lambda phage strains developed in the current studies in the parental and immunocompromized B-cell deficient mice developed by Srivastava et al. (2004). It is curious that a single phage mutation, resulting in a substitution of the basic amino acid lysine for the acidic amino acid glutamic acid results in at least three orders of magnitude greater capacity to remain in the mouse circulatory system, 24 h after i.p. injection.

Could the λ capsid E protein with a lysine at position 158 represent a protein of the capsid found in λ in the phage's "native" environment? In this regard, it should be noted that the parental "wild type" phage is derived from phage strains that have been plaque isolated and cultured under laboratory conditions for decades.

In addition to the use of these isogenic λ phage strains for studies of the innate immune response, the correlation noted in prior studies between the ability of phage to remain in the circulatory system and their ability to rescue bacteremic animals suggests that information concerning the enhancement in the "long-circulation" capacity of phage strains could be useful for antibacterial therapy, drug delivery and gene therapy (Merril et al., 1996).

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